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Malaria is the world's most ravaging infectious disease. It is rampant throughout much of the tropics and some of the temperate areas of the world. It threatens a third of the world's population, presently afflicting hundreds of millions of people, causing several million deaths annually and possibly generating as many as 92 million new clinical cases each year. The socio-economic drain of the disease is enormous.

Resurgence of this pestilence during the past 20 years due to global warming, drug resistance, and other factors has prompted increased research efforts in the production of an effective vaccine. In the interim, there must be a reliance on chemotherapy for prevention and treatment of the disease.

The current research addressed the need to develop a broad-spectrum antimalarial. Research efforts focused on the propagation of malarial parasites in vitro for the purpose of studying the unique biochemistry of the parasite with the goal of chemotherapeutic exploitation.

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Linda Nolan 11/26/97

PI - Signature Date

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Introduction

Malaria is the world's most ravaging infectious disease. It is rampant throughout much of the tropics and some of the temperate areas of the world. It threatens a third of the world's population, presently afflicting hundreds of millions of people, causing several million deaths annually and possibly generating as many as 92 million new clinical cases each year. The socio-economic drain of the disease is enormous.

Resurgence of this pestilence during the past 20 years due to global warming, drug resistance, and other factors has prompted increased research efforts in the production of an effective vaccine. In the interim, there must be a reliance on chemotherapy for prevention and treatment of the disease.

The current research addressed the need to develop a broad-spectrum antimalarial. Research efforts focused on the propagation of malarial parasites *in vitro* for the purpose of studying the unique biochemistry of the parasite with the goal of chemotherapeutic exploitation.

Review of malaria culturing techniques

Introduction:

Many biochemist, botanists, clinicians, and ethnobiologists have expressed interest in testing medicinal compounds or plant extracts against malarial parasites. Our research centered on a review of the basic techniques and the necessary equipment for the establishment and maintenance of malarial cultures. From reports in the literature we developed our own protocol designed to screen potential antimalarials.

P. falciparum cultures can be easily maintained in plastic flasks for months or years. The culture flasks provide a constant source of parasites and the results of *in vitro* experiments should be readily comparable. While some researchers have suggested that long-term *in vitro* maintenance may change the characteristics of the organism, we found that over the space of a year, both strains of *P. falciparum* maintained a constant level of susceptibility to chloroquine and pyrimethamine. To compare the effectiveness of various compounds as antimalarials, they were compared to the ability of cholorquine and pyrimethamine to suppress a parasitic infection.

The world's foremost parasitic killer, malaria affects more than 800 million people each year and may be responsible for as many as two million deaths. Malaria includes four known organisms, two of which are responsible for most of morbidity and mortality associated with the disease. *Plasmodium falciparum* has the highest level of mortality and is responsible for approximately 300 million cases worldwide and 1-2 million deaths, many of them children. (1) *Plasmodium vivax* has a lower mortality rate but a high level of reinfection. *Plasmodium ovale* and *Plasmodium malafiae* are rarely seen in clinical setting and may be better adapted to nonhuman hosts.

Malaria is transmitted by the *Anopheles* mosquito. When it bites a human for a blood meal, it releases anticoagulant from its salivary gland to prevent clotting. The salivary fluid in an infected mosquito also contains sporozoites that migrate though the bloodstream and invade parenchymal hepatocyte. After approximately one hour the sporozoites are undetectable in the bloodstream. (2)

It is worth noting that not all sporozoites are alike; some are immediately active and begin colonization and replication while others (hypnozoites) can remain quiescent in the liver for months or years before causing "relapses" of asexually replicating by producing many schizonts from each cell. It has used most of the hemoglobin in the red blood cell that causes the cell to begin to contract until it is burst by exiting merozoites that seek out and infect additional cells.

On penetrating the erythrocyte not all merozoites continue the process of becoming a trophozoite and undergoing fission. Some differentiate into single gametocytes; either micro-gametocytes or macrogametes. (2) The sexual cycle of reproduction occurs in the *Anopheles* mosquito. These parasites remain in erythrocytic capsules until the cell wall is lysed by the digestive juices of the stomach which release the gametes, which are unaffected by the digestive enzymes, into the lumen of the stomach. In the stomach lumen the microgametes exflagellate whereby the nucleus undergoes mitotic division, producing 6-8 nuclei each of which ends up independently swimming to the macrogamate which has deployed a membrane fertilization cone to be penetrated by the microgamete. This syngamy produces a zygote which quickly matures into an ookinete which migrates into the gut wall of the mosquito and becomes an oocyst. 10 - 24 days after the mosquito has ingested the gametocytes, sporozoites rupture the oocysts and

migrate to the salivary glands, to be injected into the next host when the mosquito takes a blood meal.

The symptomology and pathogenesis of malaria infection is related to the parasite's stage of growth and the host's parasitemia. High parasitemias result in decreased hemoglobin and a lower oxygen carrying capacity. This quickly leads to fatigue and repository distress. The detritus of lysed red blood cells as well as the explosion of foreign bodies in the bloodstream from logarithmic growth of malaria results in massive immune responses, primarily driven by mononuclear phagocyte released cytokines. Since the explosions of parasitemia occur in regularly timed waves (from as little as 36 hours in *P. falciparum* to 72 hours in *P. malatiae*), the characteristic periodic fever develops. The fever is a response to the massive interferon and interleukin release which subsides as the body clears the infection and the merozoites invade new cells to begin a new cycle.

The systemic damage of malaria is not only from septicemic reactions to cytokines, but also the proliferation of knobs on the red blood cell surface, sometimes called pigmotic cells. These projections can result in agglutination of parasites at capillary pathways and ischemia to many organ area. Untreated infections can lead to splenomegaly and, in *P. falciparum*, to cerebral malaria and death. Children are particularly susceptible to cerebral malaria and the bush doctor cure for urgent cases which is an immediate blood transfusion from a close member of the family has resulted in a high rate of pediatric AIDS in Africa (3).

Preparing to Culture *P. falciparum*:

The medium:

To prepare for the maintenance of *P. falciparum* cultures an appropriate medium needs to be prepared. Based on the work of Traeger and Jensen (4), the following is a standard preparation that was used to maintain cultures in our laboratory: *P. falciparum* cultures are initiated and maintained in RPMI 1640 media supplemented with glucose, TES, pyruvate, 1.1% (w/v), glutamine, 3% (w/v), hypoxanthine, .05% (w/v), Gentamicin, and type A+ human plasma.

Unsupplemented RPMI 1640 media is commercially available in 500 ml bottles (or it may be reconstituted from powder). We use 500 mL bottles from which 75 mL have been withdrawn. For each 500 mL bottle of RPMI 1640 (now containing 425 mL), add the following:

In a 50 ml centrifuge tube, combine:

1 g glucose

1.5 g TES

5 mL pyruvate 1.1 % (w/v)

5 mL glutamine 3% (w/v)

5 mL hypoxanthine 0.05% (w/v)

Vortex the centrifuge tube to mix the contents. This part of the process does not need to be performed under sterile conditions. To sterilize the contents of the centrifuge tube before addition to the media, filter it through a 0.2 AM syringe filter attached to 30 mL syringe as it is added to the RPMI 1640 bottle. To avoid bacterial contamination of the media, add 0.1 mg/mL Gentamicin.

Finally, add 50 mL of centrifuged human plasma to the media. While our laboratory used A+ red blood cells and plasma because it was readily available and economical, *P. falciparum* does not seem to exhibit any preference. The media should ideally be prepared 48 hours in advance, so several mL can be transferred to a small flask and incubated at 37°C for 48 hours to check for contamination. If there is a problem, there will be colonies of fungi or bacteria visible to the naked eye. Store the supplemented RPMI 1640 media at 4°C.

Our laboratory used type A+ human blood to maintain cultures. The blood should be transfusion grade packed red blood cells (RBC) and as fresh as possible. While *P. falciparum* does not invade old RBC well, our laboratory successfully used blood up to three weeks after the clinical use expiration date without problems. Note that the older RBCs can be an additional source of stress and cultures may be more prone to crash in expired blood.

Preparation of RBCs: Washing the blood

1) Aliquot the blood into 15 mL centrifuge tubes using a fresh plasma transfer kit. Ideally the plasma transfer kit is introduced into the transfusion bag under sterile conditions. It is very important to clean the port of the transfusion bag into which the plasma transfer applicator will be placed. The needle end of

the kit shouldbe used to dispense 12 mL of blood into the tubes. After closing the line and capping the needle, place the entire setup in a 4°C refrigerator.

2) Add RPMI media with ONLY the gentamicin added, to fill the centrifuge tube to the 14.5 mL mark. Mix well - DO NOT vortex - and centrifuge for 10 minutes at 2000 rpm. Aspirate the media down to and including the buffy coat of white blood cells and excess material. Repeat the wash. Aspirate the media and resuspend the cells in the same RPMI media with Gentamicin only, at a final 50% hemocrit - add 1 mL of RPMI media for every 1mL of cells.

Establishing and maintaining cultures:

Our laboratory found it most cost efficient and easiest to maintain the cultures in 25 cm^3 flasks. These allow easy maintenance using less media while still allowing a large enough parasite population to coat a 96-well microtitre plate. To concentrate cultures for enzyme studies or to run assays in several 96-well plates simultaneously, larger flasks should be used:

Table 1. Procedure for establishing a 1% parasitemia in various sized flasks:

Flask Size	25 cm^3	75 cm^3	150 cm^3
Supp. Media	5 ml	25 ml	50 ml
Washed RBCs	0.5 ml	2.5 ml	5 ml
Gas (seconds)	20s, 3-4 PSI	40s, 6-8 PSI	60s, 6-8 PSI

The parasitemias of the culture flask was maintained at around 1 % to 5 %. Higher parasitemias for periods longer than 36 hours resulted in stressed cultures with large numbers of dead parasites and misshapen RBCs. Healthy parasite cultures will grow from 1% to 8% in approximately 42 hours depending on the strain of parasite - W2 seemed to grow much quicker with a ring-to-ring cycle of around 39 hours and HB3 was more gradual with ring-to-ring times around 44 hours. Parasitemias in culture of 10% to 17% are possible for short periods of time but result in very stressed environments with stippled RBCS. The highest parasitemia we achieved was 23%. After this point, the amount of debris and the condition of RBCs and media made evaluation impossible. While clinical reports vary, sources rarely cite human parasitemias greater than 15%, even during periods of synchronous outbreak at the height of the 48 hour cycle.

Assessing Parasitemia:

To monitor the actual parasitemia of the culture, slides were taken during each media replenishment to determine how to split the culture. The Diff-Quik stain kit (Baxter-VWR, Philadelphia, PA) was used to stain the slides. This procedure involves removing the excess media which sits above the RBCs in the culture flask. This can usually be done by slightly tilting the flask and aspirating the media at one end. Then a pasteur pipette is used to collect a drop of parasitized RBCs to be placed on a slide for a smear sample. The slide should already have been labeled at one end. It is important to smoothly and evenly run the edge of another slide across the drop of blood on the slide to be examined. Rough, jerky movements will result in uneven layers of thickness and inaccurate counts. The slide can than be air dried for approximately 20 seconds, until the patina of the blood smear changes.

Using the Diff-Quik stain kits, the slides (without covers) are immersed in the fixing agent for 5-7 seconds. (Note that the fixative solution will remove and smear permanent marker, so do not totally immerse the slide.) Then the slide should be put in solution I for NOT more than 20 seconds and solution II for 25-30 seconds. Do not keep the slide in the air for more time than necessary between solutions. Thinner smears seem to be more readable if less time is spent in solution II. Slides should then be briefly placed under running cold water, blotted on paper towel, and air dried. Parasitemia can then be assessed by counting 1000 or 2000 parasites, depending on the level of accuracy needed. Our laboratory counted 1000 RBCs, and our slides usually had 200 RBCs per field. Note that counting requires an oil immersion 100x lens and that an orange or blue filter can make parasite resolution easier with the Diff-Quik stains, especially to include the faint early ring stage.

Assessment pitfalls and staging:

There are four common difficulties in assessing slides. Water, usually from the humid air or Diff-Quik solution which is too old or contaminated with too much fixing agent, can produce many false RBCs.These are round and multi- chromatic. Too much blood on the slide results in multiple layers of RBCs which are generally uncountable because they are superimposed on each other. Staining the slides too dark or too light results in pale orange or dark purple slides that do not allow the delicate ring structure to be accurately counted. Finally, a careful pattern must be rigorously held to and universally applied, usually by the same person assessing slides to be compared.

For a simple parasitemia, the number of infected RBCs would suffice. However our laboratory always broke the parasitemia into four stages: ring, late ring, trophozoite and schizont. With some familiarity with a particular strain, the breakdown of various stages coupled with the natural tendency of plasmodium to synchronize its life cycle, will allow an extra check of the health and status of the culture.

The initial culture:

Place a small amount of infected RBCs, from whole blood or another culture, in a 25 cm^3 flask with 5 mL of supplemented RPMI media and 0.5 mL of washed, packed RBCs.

Culture maintenance:

Remove the culture from the incubator making sure not to agitate it severely. It is important to note the color of the culture as the oxygenation of the blood is a rough indication of the parasites metabolism and health status. With a sterile pipette, aspirate the excess media off the RBCS, remove a drop of blood to a slide and assess the parasitemia. While the slide is air drying, add 5 ml of supplemented RPMI media to the culture, seal it and stand it up to disperse the new media. If the parasitemia is low, around < 1%-2%, then add 0.1 ml of RBC if necessary.

If the parasitemia is higher, the culture should probably be reduced to the 1%-2% range. This is done by agitating the flask to distribute the parasitized RBCsin media and use a IO ml pipette to remove all the media from the flask. If the culture was to be split 1:5, then return 1 ml of infected RBCs and distribute the rest to new cultures or into a flask of Wescodyne or other appropriate antimicrobial solution. In the 1:5 split, 4 ml of supplemented media would then be added, along with 0.5 ml of washed RBCS.

Table 2. Guidelines for maintaining a 1%-2% parasitemia in 25 cm^3 culture flask.

Percent (%) Parasetimia	Split Ratio	Parastized RBCs (mL)	Supplemented Media (mL)	Washed RBCs (mL)
1-2	none			0.1 if needed
3-4	1:3	1.5	3.5	0.5
5-8	1:5	1.0	4.0	0.5
8-12	1:10	0.5	4.5	0.5
> 12	1:20	0.025	4.75	0.5

Creating a proper atmosphere:

The gaseous environment of cultures was maintained at 5% CO_2, 1% O_2, 94% N_2. This was created by introducing a premixed gas at low pressure into the flask. A pipette with a cotton filter was used to distribute the gas equally across the surface of the culture. The flasks were then tightly sealed and placed in a 37°C incubator.

An alternative method for creating the proper environment that avoids the need for premixed gas canisters was to place the culture flasks or microtitre plates in a candle jar with a lit candle. In this case the flask tops are not screwed on but simply placed over the openings. This should allow oxygen from inside the flask to escape toward the candles, while avoiding the introduction of particulate from the burning candle. It is important to leave the candlejar top ajar until the point immediately after the candle has gone out. Then the lid should be quickly closed and the candle jar itself placed in a 37°C incubator.

Materials:

Plasmodium falciparum strains HB3 (from South America) and W2 (from Indochina) were generously provided by Dr. D. Wirth, School of Public Health, Harvard Medical School, Boston, MA.

Large-Scale Cultivation of *Plasmodium falciparum* for Enzyme Analysis

Despite decades of research and applied management, malaria still ranks as one of the most widespread infectious diseases in the world. As the numbers of drug-resistant strains of malaria parasites increase, the need for new chemotherapeutic agents intensifies. Recent studies suggest targeting *Plasmodium falciparum* enzymatic pathways in the development of new anti-malarial compounds. However, this avenue of research requires high enzyme concentrations. Therefore, the *in vitro* production of relatively large numbers of specific parasite stages expressing enhanced enzyme activity is desired. A combination of culturing protocol and harvesting technique has been developed which optimizes the production of erythrocyte parasitemia without chemical intervention and allows the separation of parasite stages for further enhancement of enzyme activity. The culturing protocol, levels of parasitemia maintained, quantification of developmental stages, and results of the separation technique for enzyme analysis are described.

Introduction

Despite decades of research and applied management- malaria still ranks as one of the most infectious diseases in the world (5). Chemotherapeutic approaches to malaria treatment are hindered by the widespread reemergance of drug-resistant strains of *Plasmodium falciparum,* the species responsible for the most virulent form of human malaria (5).

Topoisomerases are essential enzymes that catalyze the concerted breaking and rejoining of the DNA backbone, processes involved in DNA replication, transcription, and recombination. These enzymes are present in high levels in rapidly dividing cells, such as cancer cells and late blood-stage (trophozoite and schizont) malaria parasites (6). Since the growth rate (and DNA synthesis rate) of the late blood stage parasites resembles that of a malignant more than a normal cell, it has been suggested that the parasites man be more sensitive to DNA-compromising drugs than normal human cells (7, 8). Numerous antibacterial and antitumor agents are already known to inhibit topoisomerases type II (9). Topoisomerases, therefore, have become targets for antimalarial chemotherapy.

Development of a rapid drug-evaluation system for screening purposes is essential. Our laboratory currently maintains two strains of *P. falciparum* of different origin and drug sensitivity Strain HB3 (Honduras) is pyrimethamine resistant and strain W2 (Indochina) is chloroquine resistant.

In Vitro Cultivation of *Plasmodium falciparum*

Establishment of Cultures:

Materials

Plasmodium falciparum strains HB3 (South America) and W2 (Indochina) were generously provided by Dr, D. Wirth, School of Public Health, Harvard Medical School, Boston, MA. RPMI 1640 medium was obtained from Gibco (Grand Island, NY). Glucose, N-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid (TES), pyruvate, glutamine, hypoxnthine, gentamicin, and human AB serum were purchased from Sigma Chemical Company (St. Louis, MO). Fresh frozen human plasma, type A +, and transfusion grade packed red blood cells, type A positive, were obtained from the American Red

Cross (Boston, MA). Tissue culture flasks with canted necks and plug seals were purchased from Fisher Scientific (Pittsburgh, PA). Diff-Quik stain kit was obtained from Baxter-VWR (Philadelphia, PA).

Methods and Conclusions

RPMI 1640 medium was supplemented with 1. 1% glucose, 1. 1% TES, 1.1% pyruvate, 3% glutanitine, 0.05% hypoxanthine, 25 µg/mL gentamicin, and 10% type A+ human plasma. Packed red blood cells (RBCS) were washed twice with unsupplemented RPMI 1640 medium containing 25 µg/mL gentamicin prior to use in culturing. Parasites were cultured in 5% hematocrit in a 5% CO_2, 1% O_2, 94% N, environment created by passing gas over cultures then storing with their caps tightly closed in a 37°C incubator. Table 3 presents the guidelines used for establishing cultures in various sized flasks.

The optimal range of parasitemia was determined to be 1% to 10% for the long-term culturing of *P. falciparum*. Healthy parasite cultures were found to grow from 1% to 10% in two days, with the ring-to-ring cycles determined to be approximately 44 and 41 hours for *P. falciparum* strains HB3 and W2, respectively. Table 4 presents the guidelines used for maintaining cultures in various sized flasks. Table 5 presents guidelines for subculturing *P. falciparum* grown in 25 cm^2 flasks. Cultures were evaluated for health and parasitemia by microscopic examination of thin lave slides. Slides were stained using the Diff-Quik stain kit as follows: 5 seconds in fixative, 20 seconds in solution I, and 30 seconds in solution II. Slides were then washed in running tap water and air dried. Parasitemia was determined by counting the number of parasites in 1000 RBCs. For assay purposes, parasitemia was determined and categorized by the following stages: ring, late ring, trophozoite, and schizont.

Table 3. Guidelines for establishing *P. falciparum* cultures in various sized flasks.

Flask size:	25 cm^2	75 cm^2	150 cm^2
Medium	5 mL	25 mL	50 mL
Washed RBCs	0.5 mL	2.5 mL	5 mL
Parasitemia	1%	1%	1%
Gas	20 sec (3-4 Psi)	40 sec (6-8 Psi)	60 sec (6-8 Psi)

Table 4. Guidelines for maintaining *P. falciparum* cultures in various sized flasks.

Flask size:	**25 cm^2**	**75 cm^2**	**150 cm^2**
Medium	5 mL	25 mL	50 mL
Washed RBCs[1]	0 mL	0 mL	0 mL
Parasitemia	1-2%	1-2%	1-2%
Gas	20 sec (3-4 Psi)	40 sec (6-8 Psi)	60 sec (6-8 Psi)

Table 5. Guidelines for subculturing *P. falciparum* grown in 25 cm^2 flasks.

		Volume (mL)		
Perencet Parasitemia	Split Ratio	Parasitized RBCs[2]	Fresh medium[3]	Fresh RBCs[4]
1-2	None			0.1 if needed
3-4	1:3	1.5	3.5	0.5
5-8	1:5	1.0	4.0	0.5
8-12	1:10	0.5	4.5	0.5
> 12	1:20	0.025	4.75	0.5

Concentration of Viable Trophozoite- and Schizont-Infected Red Blood Cells

Materials

Plasmagel was purchased from Cellular Products, Inc. (Buffalo, NY).

Methods

Cultures were suspended in medium, transferred to centrifuge tubes, and centrifuged for 5 minutes at 800 X g. The pellet was resuspended in unsupplemented medium containing 25 µg/ml

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[1] Every other day sufficient RBCs were added to maintain 5% hematocrit, if necessary.

[2] Volume of parasitized RBCs/media left in culture flask.

[3] Volume of fresh, supplemented medium added to culture flask.

[4] Volume of fresh, washed RBCs added to culture flask.

gentamicin and centrifuged for 5 minutes at 800 X g. Supernatant was removed and unsupplemented medium containing 25 µg/mL gentamicin was added to reach a final hematocrit of approximately 40%. Plasmagel was added to the cell suspension at a ratio of 2:1 cell suspension: plasmagel and gently mixed. The suspension was incubated for 15 min at 37°C,. The upper phase was transferred to a centrifuge tube and centrifuged for 10 min at 800 X g. The pellet containing the concentrated late stage parasites was resuspended in supplemented medium, slides were made, and parasite stages counted.

Conclusions

Results of a typical plasmagel separation of parasite stages for *P. falciparum* strain W2 are presented in Table 6. The initial culture had a relatively uniform distribution of parasites stages with a combined trophozoite + schizont percentage of 43.0. The plasmagel methodology resulted in the isolation of a subpopulation of parasites with a combined trophozoite + schizont percentage of 82. 0.

Table 6. Results of a typical plasmagel separation of parasite stages for *P. falciparum* strain W2.

% of total Parasitized RBCs:	Early stages (rings/late rings)	Late stages (trophozoites/schizonts)
Initial culture	57	43
Upper Phase	18	82

Crude Extract Preparation for Enzyme Assays

Materials

Kinetoplast DNA, supercoiled (form 1) plasmid DNA, topoisomerase types I and II assay buffers (10X), and human topoisomerase types I and II were obtained from TopoGEN, Inc. (Columbus, OH).

Methods

Preparation of parasite extracts: The following is a modification of the methodology of Inselburg et al. (10). Asynchronous populations of parasitized RBCs were suspended in culture medium, centrifuged for 10 minutes at 800 X g, and resuspended in 3 ml phosphate buffered saline (PBS, 0.01 M KH_2PO,/K_2PO_4, 0. 14 M NaCl, pH 7.4). Additional PBS was added and the cell suspension was centrifuged for 10 minutes at 800 X g. The supernatant was removed and volume of pellet was recorded. The pellet was resuspended in 20 volumes of (room temperature) 0.01 5 % saponin in PBS, incubated at room temperature for 10 minutes with occasional mixing, then centrifuged for 10

minutes at 5000 X g at 4°C. Supernatant was removed and the pellet was washed 2 X with cold PBS. Parasites were resuspended at a concentration of 1.7 X IO^9 parasites/ml in TE buffer (50 mM Tris, 1 mM EDTA, pH 7.4) containing 10 mM benzamidin, 20 µg/mL leupeptin, 20 µg/mL pepstatin, 50 µg/mL aprotinin, and 50 µg/ml soybean trypsin inhibitor, sonicated in an ice bath with 5 equally spaced 10 second bursts at full power during a 4 minutes period, then centrifuged for 30 minutes at 29,900 X g at 4°C. The supernatant was stored at -80°C.

Topoisomerase type II decatenation activity: Topoisomerase type II decatenation assays were conducted using kinetoplast DNA (kDNA), the mitochondrial DNA as the DNA substrate. Reaction mixtures containing assay buffer (50 mM 1'ris-HCL, pH 8.0, 120 mM KCI, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithlothreltol, 30 μg/mi nuclease free BSA), 275 ng kDNA, and 5 μl extract in a total volume of 20 μl, were incubated at 37°C for 60 minutes. Human topoisomerase type II was used as the positive control. At the end of 60 minutes, 1 μl (containing 1. 8 μg) proteinase K was added for a final concentration of 50 μg/mL. Samples were incubated at 37°C for 15 minutes. Reaction mixtures were stopped by the addition of 2 μl of gel loading buffer containing 0.25% bromophenol blue in 50% glycerol. Twenty μl of chloroform:isoamyl alcohol (24:1) were added, samples vortexed briefly, then centrifuged for 5 seconds in a microfuge.

Topoisomerase type I relaxation activity: Topoisomerase type I relaxation assays were conducted using supercoiled (form 1) plasmid DNA as the DNA substrate. Reaction mixtures containing assay buffer (10 mM Tris-HCI, pH 7.9, 1 mNi EDTA, 0.15 M NaCl, 0.1% BSA, 0. 1 mM spermidine, 5% glycerol), 0.25 μg DNA, and 5 μl extract in a total volume of 20μl, were incubated at 37°C for 60 minutes. Human topoisomerase type I was used as the positive control At the end of 60 minutes, reaction mixtures were stopped by the addition of 2 μl of gel loading buffer containing 0.25% bromophenol blue in 50% glycerol. Twenty μl of chloroform:isoamyl alcohol (24:1) were added, samples vortexed briefly, then centrifuged for 5 seconds in a microfuge.

Gel electrophoresis: Eighteen μl of the blue colored upper phase were electrophoresed through 0.9% agarose gels for 2 hours at 25 mA current with a forward pulse time of 6 seconds, a reverse pulse time of 2 seconds, and a ramp setting of 1. The running buffer was 0.5 X TBE (89 mM Trisbase. 89 mM boric acid, 2 mM EDTA, pH 8.2).

Discussion

The creation of an effective, antimalarial drug-evaluation system requires the development and incorporation of a variety of different methodologies. Our laboratory has established and routinely maintains two strains of *Plasmodium falciparum* of different origin and drug sensitivity, the pyrimethamine-resistant strain HB3 from Honduras and the chloroquine-resistant strain W2 from Indochina.

In addition, a rapid, crude enzyme extraction preparation has also been developed. Both topoisomerase type I and II activities are present in these extracts, which provide a tool for drug inhibition studies (fig. 1 and fig. 2).

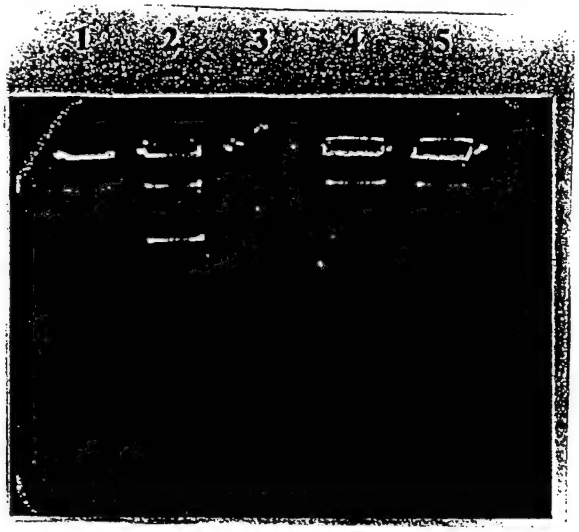

Figure 1: Results of topoisomerase type II decatenation assay using crude extract of *P. falciparum* strain HB3.
Lane 1: kDNA control in reaction mixture without enzyme;
Lane 2: decatenation activity of human topoisomerase type II;
Lane 3: *P. falciparum* extract in reaction mixture without kDNA substrate;
Lane 4: decatenation activity of *P. falciparum* extract;
Lane 5: heat-activated (10 minutes at 50°C) *P. falciparum* extract with kDNA substrate.

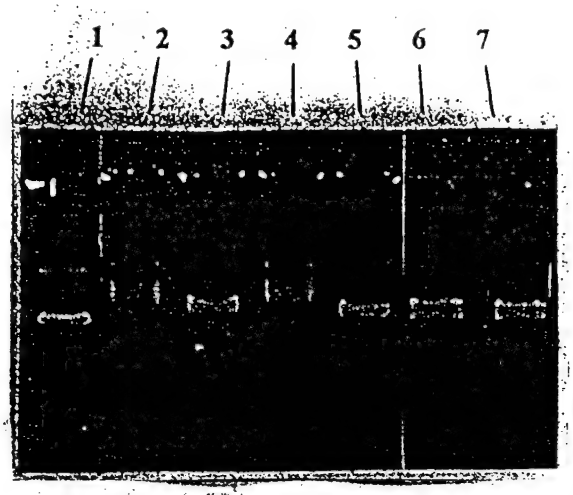

Figure 2: Results of topoisomerase type I relaxation assay using crude extracts of *P. falciparum* strains HB3 and W2.
Lane 1: supercoiled plasmid DNA control;
Lane 2: relaxation activity of *P. falciparum* strain HB3 extract;
Lane 3: heat-activated *P. falciparum* strain HB3 extract with supercoiled DNA substrate;
Lane 4: relaxation activity of *P. falciparum* strain W2 extract;
Lane 5: heat-inactivated *P. falciparum* strain W2 extract with supercoiled DNA substrate;
Lane 6: relaxation activity of human topoisomerase type I;
Lane 7: relaxed marker DNA.

Preparation of *Plasmodium falciparum* Crude Extract for Topoisomerase Type I and II analysis: Preliminary Methodology Experiments

Materials

Kinetoplast DNA, topoisomerase type II assay buffer (10 x), and human topoisomerase type II were obtained for TopoGEN, Inc., (Columbus, OH). Dye reagent for protein assay was purchased from Bio-Rad Laboratories, Hercules, CA.

Preparation of parasite extracts

Preliminary experiments were conducted on asynchronous parasite cultures of *Plasmodium falciparum* strains HB3 and W2 (Table 7). Populations of Parasitized red blood cells (RBCs) were suspended in culture medium, centrifuged, and washed once with Hanks Balanced Salt Solution (Sigma Chemical Co., St. Louis, MO). After the supernatant was removed, the cells were immediately frozen in liquid nitrogen and transferred directly to a -80°C freezer for storage. RBCs not infected with parasites were also processed in the same manner to serve as experimental controls.

Removal of hemoglobin

The following procedure is a modification of the methods of Yohida and Watnabe (11) and Colowick and Kaplan (12). Frozen RBC pellets were allowed to thaw on ice. Pellets collected on different dates were combined into one sample for each strain, total wet weights were determined (Table 8). An equal volume of 0.01 M phosphate buffer, pH 7.5, was added to each sample. Samples were vortexed and aliquots were removed for protein analysis. 24 μl of ethanol-chloroform (2:1, v/v), cooled at -60°C, was added to each 100 μl of sample. Samples were shaken vigorously on ice for 20 minutes, then centrifuged at 13,000 x g for 30 minutes. The hemoglobin precipitates were discarded and aliquots of the supernatants were removed for protein analysis. The supernatants were then divided into two aliquots. One was used directly in the enzyme assay, the other was sonicated 3 x 2 seconds on ice, prior to enzyme analysis.

Protein analysis

Protein was determined according to a modified bio-Rad dye-binding method. Assays were conducted in Falcon 3071 microplates (Fisher Scientific, Pittsburgh, PA) and analyzed by a Molecular Devices' THERMOmax microplate reader (Menlo Park, CA) at 590 nm.

It has recently been suggested that topoisomerases could be important targets for drugs used in several diseases. This prompted us to purify and characterized the topoisomerases I and II present in the erythorocytes of malarial parasites.

Topoisomerase type II decatenation activity

The topoisomerase type II decatenation assay was used as an initial screen for enzyme activity due to its sensitivity for detection of low enzyme titers (TopoGEN, 1995). Topoisomerase type II decatentation assays were conducted using kinetoplast DNA (kDNA), the mitochondrial DNA of *Crithidia fascuculata*, as the DNA substrate. Reaction mixtures containing assay buffer (50 mM Tris-HCL, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0m5 mM dithiothreitol, 30 μg/ml nuclease free BSA), 275 ng kDNA, and 5 or 10 μl crude extract in a total volume of 20 μl, were incubated at

37°C for 60 minutes. Human topoisomerase type II was used as the positive enzyme control. Reaction mixtures were stopped by the addition of 2 μl of gel loading buffer containing 0.25% bromphenol blue in 50% glycerol. Twenty μl samples were electrophosered through 0.9% agarose gels for 2 hours at 25 mA current with a forward pulse time of 6 seconds, a reverse pulse time of 2 seconds, and a ramp setting of 1. The running buffer was 0.5 x TBE.

Conclusions

Total protein values for the crude extracts are presented in Table 3. The protein content of the crude extracts used in the enzyme assay (for extract volumes of 5 and 10 μl, respectively) are: 530 and 1060 μg for strain HB3; 155 and 310 μg for strain W2; and 300 and 600 μg for control RBCs.

Decatenation activity was not observed in any of the crude preparations. Decatenation of kDNA was observed in the presence of human topoisomerase type II. (kDNA, although not present in these parasites or in human cells, is a good substrate for the enzyme being tested when compared to other DNA types.)

We plan to increase the parasitemia of synchronized cultures to increase protein yield and enzyme activities. Also a new protocol to remove hemoglobin will be tested to see if we can enhance enzymatic activity.

We will also establish a routine protocol to evaluate a range of antimicrobial agents against *P. falciparum* cultures. A modification of the semiautomatic method of Desjardins (13) will be used

Table 8. Percents parasitemia and life stages of *P. falciparum* cultures used in crude extract preparations.

Strain	Percent Parasitemia	Percent Rings	Percent Later Stages (Trophozoites & Schizonts)
HB3	10-15	50	50
W2	22	40	60

Table 9. Total wet weights of combined pellets used in crude extract preparations of *P. falciparum* strains.

Strain	Wet Weight (grams)
Strain HB3	0.7750
Strain W2	0.8610
Control RBCs	0.1289

Table 10. Total protein values obtained for steps in the crude extraction preparation.

	Total Protein (mg)		
Extraction step	HB3	W2	Control RBCs
Resuspended pellet	287	277	27
Hemoglobin removed	26	1.2	4.1

Testing Compounds for Antimalarial Activity

Objective

To determine the inhibitory effects of test compounds on the growth of *P. falciparum* parasites.

Materials and Methods

WRAIR Test Compounds: The following compounds were received from WRAIR. They were dissolved according to the suggestions and the molecular structures on the data sheet sent by WAIR:

Receiving Date: November 7, 1996

WR. No.	Bottle No.	Solvent
006026	BK01845	RPMI assay medium
238605	BL19664	RPMI assay medium
242511	BM04186	RPMI assay medium

Other test compounds: The following compound were received from Pfizer Incorporated. It was dissolve according to the suggestion and molecular structures on the data sheet received from Pfizer Inc.:

Receiving date: December 13, 1996

Compound	Lot#	Solvent
Fluconazole	24P-900-E	RPMI assay medium

Parasites and Medium: The compounds were tested against *Plasmodium falciparum* strain HB3 (S. America, pyrimethamine resistant). Tests used RPMI 1640 medium (from GIBCO BRL) supplemented with glucose, TES, pyruvate, glutaminutese, Gentamicin, and type A+ human plasma.

Compound Tests

48 hour screen tests were conducted in 96-well plates. Parasite growth was measured by comparing the incorporation of ^{3}H-hypoxanthine into parasite DNA of controls with treated wells.

Microplate Protocol: 25 μl of the test compounds was added to each well followed by 200 μL parasite culture (asynchronous; diluted to 1% parasitemia). Control wells consisted of untreated infected red blood cells. Blank well had only 200 μL normal red blood cells or 200 μL medium. After 18 to 20 hours of incubation, 0. 5 μCi 3H-hypoxanthine was added to each well. Wells were processed after an additional 26 to 28 hours of incubation. Test compounds were assayed in duplicate.

Well processing: After the final incubation, well contents were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 minutes at 1500g. The supernatant was removed and the cells washed twice (centrifuging for 5 minutes at 15OOg between washes) with ice-cold saline. 500 μL distilled H_20 was added, and the tubes vortexed until all cells were lysed. 200 μL hemolysate was transferred to 5 mL scintillation fluid and vortex until thoroughly mixed.

Conclusions

Compound:		BK01845		BM04186		BL19664		Flucanazole
	conc.	% of control*	conc.	% of control*	conc.	% of control*	conc.	% of control*
	0.05 μM	86	0.1 μM	65	0.1 μM	85	0.1 μg/ml	93
	0.5 μM	27	0.5 μm	18	0.5 μM	73	0.5 μg/mL	92

Compound:		BK01845		BM04186		BL19664		Flucanazole
	1 μM	14	2.5 μM	4	2.5 μM	29	2 μg/mL	100
	2.5 μM	9	7.5 μM	2	7.5 μM	13	5 μg/mL	87
	10 μM	4	15 μM	1	15 μM	7	12 μg/mL	78
conc.range (ug/mL)	0.02-4.2		0.05-7.8		0.07-10		0.1-12	
IC_{50}		0.2 μM		0.25 μM		2 μM		>12 μg/mL

* % IC is % of control

Remarks

All three of the compounds inhibited the growth of *P. falciparum*. BK01845 and BM04186 are of note with the low concentrations required to elicit the IC_{50}. Correspondence received during the Leishmania assays indicates maximum human serum levels have been established for BKO1845 and its major metabolite, WR211789 (BK50713 -- not one that we have at the moment) at 104 μg/mL and 25 μg/mL respectively. The IC_{50} of BKO845 is MUCH lower less than 0.3 μg/mL. I do not know what studies have been carried out by WRAIR on this compound. However, if BKO1845 has not already been closely examined for anti-malarial properties, it warrants further analyses

The Pfizer Inc. compound, Fluconazole, did not have a noticeable inhibitory effect until the concentration approached 12 μg/mL.

Receiving date: November 7, 1996

W.R. no.	Bottle No.	Solvent
242511	BM04186	RPMI 1640 assay medium
238605	BLI9664	RPMI 1640 assay medium
006026	BKO1845	RPMI 1640 assay medium

Parasites and Assay Medium: The compounds were tested against the W2 strain (Indochina, chloroquine resistant) of *Plasmodium falciparum*.

For two days prior to the screening test and throughout the tests, parasites were cultured in RPAE 1640 medium (from GIBCO BRL) supplemented with glucose, TES, glutaminutese, Gentamicin, pyruvate, and type A+ human plasma.

Testing Process

48 hour screening tests were conducted in 96-well plates. Parasite growth was determined by comparing the incorporation of ^{3}H-hypoxanthine into parasite DNA of controls vs. ^{3}Hhypoxanthine incorporation into the DNA of the treated parasites. Results are reported as percent inhibition of control. Microplate preparation: 25 μL of the test compound was added to the appropriate well followed by 200 μL parasite culture (asynchronous; diluted to approximately 1% parasitemia). Test wells were run in duplicate. Controls consisted of 200 uL parasite culture without the addition of any test compound. Blank wells contained either 200 uL untreated, non-nal red blood cells (unparasitized) or 200 μL assay medium. The plate was sealed in a Tupperware® container flushed with gas and incubated for approximately 24 hours. After 24 hours of incubation, 0 5 μCi ^{3}H-hypoxanthine was added to each well. The container was then flushed with gas and replaced in the incubator for an additional 24 hours.

Well Processing

Analysis of bound ^{3}H-hypoxanthine: Each wells' contents were transferred to a 1.5 mL microcentrifuge tube and centrifuged for 5 minutes at 1500g. The supernatant was then removed and discarded. The red cell pellet was then washed twice with 500 μL ice-cold normal saline (0.9% NaCl). centrifuging for 5 minutes at 15OOg between each wash. The final wash supernatant was removed and the red cells lysed by the addition of 500 μL deionized water. 200 μl of the resulting hemolysate was transferred to 5 mL scintillation fluid, vortexed thoroughly, allowed to stand in cool, dark place for 30 minutes prior to counting.

Conclusions

Compound	chloroquine		BM04186		BL19664		BK01845	
	conc.	%IC*	conc.	%IC*	conc.	%IC*	conc.	%IC*
	1.0 nM	104	0.1 μM	86	1.0 μM	85	0.1 μM	100
	2.5 nM	107	0.25 μM	68	1.5 μM	77	0.25 μM	84
	10 nM	105	0.5 μM	43	2.0 μM	68	0.5 μM	65
	25 nM	98	1.0 μM	19	3.0 μM	47	1.0 μM	43
	50 nM	73	2.5 μM	14			2.5 μM	18
			0.05 - 1.3 μg/ML		0.67 - 2.0 μg/mL		0.04-0.42 μg/ML	
IC_{50}			0.48 μM (0.25 μg/mL)		2.9 μM (1.96 μg/mL)		0.85 μM (0.35 μg mL)	

*% of control

All of the IC_{50} values for W2 are slightly higher than those determined for the HB3 strain of *P. falciparum*. However, even so, all of the test compounds had IC_{50} values low enough to possibly be of therapeutic interest.

Objective

To determine the inhibitory effects of test compounds to the growth of *Plasmodium falciparum* parasites.

Materials and Methods

Test Compounds: The following compounds were received from WRAIR. They were dissolved according to the suggestions and the molecular structures on the Data Sheet sent by WRAIR and stored at 40°F:

Receiving Date: December 10, 1992

Preparation Date: January 4, 1993

Malarial Assay Date: April 9,1997

WR. No.	Bottle No.	solvent	Stock Concentration
230330	BH32724	DMSO	20μM
247733	BJ52043	DMSO	4 μM, 40 μM
074086	AH95665	DMSO	17μM, 170 μM
098657	AF55410	DMSO	25 mM

Parasites and Medium: The compounds were tested against *Plasmodium falciparum* strain HB3 (S. America, pyrimethamine resistant). Assay medium consisted of RPMI 1640 medium (from GIBCO BRL) supplemented with glucose, TES, pyruvate, glutaminutese, Gentamicin, and type A+ human plasma.

Compound Tests

48 hour screening tests were conducted in 96-well plates. Antimalarial efficacy was determined by comparing the incorporation of ^{3}H-hypoxanthine into parasite DNA of controls versus treated wells. Microplate Protocol: The maximum amount of DMS0 that can be added to parasite cultures has been established to be 0. 17% of the total volume. Two milliliter aliquots of the parasite suspension (asynchronous; diluted to 1% parasitemia) were treated with 3.4μL of the appropriate DMSO solution. Microplate wells contained 200 μL of the treated parasite culture and were run in triplicate. Control

wells consisted of infected red blood cells plus DMSO. After approximately 24 hours of incubation, 0. 5 μCi ^{3}H-hypoxanthine was added to each well. Wells were processed after an additional 24 hours of incubation.

Well Processing: After the final incubation, well contents were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 minutes at 1500g. The supernatant was removed and the cells washed twice (centrifuging for 5 minutes at 15OOg between washes) with ice-cold saline. 500 μL distilled H_20 was added, and the tubes vortexed until all cells were lysed. 200 uL hemolysate was transferred to 5 mL scintillation fluid and vortex until thoroughly mixed.

Conclusions

Compound	**Stock Conc.**	**Assay Conc.**	**Percentage of Control**
BH32724	20 μM	34 nM	107
BJ52043	4 μM	6.8 nM	97
BJ52043	40 μM	68 nM	5
AH95665	17 μM	28.9 nM	4
AH95665	170 μM	289 nM	4
AF55410	25 mM	42.5 μM	14

Remarks

With the exception of BH32724, the WRAIR compounds showed significant inhibition of parasite growth. Delineation of the IC_{50} for these compounds is in progress

Receiving Date: December 10, 1992

Preparation Date: January 4, 1993

Malarial Assay Date: April 17, 1997

WR No.	Bottle No.	Solvent	Stock Concentration
029252	AJ32190	DMSO	24.2 mM, 242 mM
029656	AJO2030	DMSO	2mM, 466 mM
180117	BGIO377	DMSO	5 μM, 50 μM
256363	BL29446	DMSO	23.1 mM
247734	BJ52052	DMSO	5 μM

230330 BH32724 DMSO 2 μM

Parasites and Medium: The compounds were tested against *Plasmodium falciparum* strain HB3 (S. America, pyrimethamine resistant). Assay medium consisted of RPMI 1640 medium (from GIBCO BRL) supplemented with glucose, TED, pyruvate, glutaminutese, Gentamicin, and type A+ human plasma.

Compound Tests

48 hour screening tests were conducted in 96-well plates. Antimalarial efficacy was determined by comparing the incorporation of ^{3}H-hypoxanthine into parasite DNA of controls versus treated wells.

Microplate Protocol: The maximum amount of DMSO that can be added to parasite cultures has been established to be 0. 17% of the total volume. Two milliliter aliquots of the parasite suspension (asynchronous; diluted to ~1 % parasitemia) were treated with 3.4 μL of the appropriate DMSO solution. Microplate wells contained 200 μL of the treated parasite culture and were run in triplicate. Control wells consisted of infected red blood cells Plus DMSO. After approximately 24 hours of incubation, 0.5 μCi ^{3}H-hypoxanthine was added to each well. Wells were processed after an additional 24 hours of incubation.

Well processing: After the final incubation, well contents were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 minutes at 1500g. The supernatant as removed and the cells washed twice (centrifuging for 5 minutes at 1500 g between washes) with ice-cold saline. 500 μL distilled H2O was added, and the tubes vortexed until all cells were lysed. 200 μL hemolysate was transferred to 5 mL scintillation fluid and vortexed until thoroughly mixed.

Assay initiated: 4/17/97 2:00 pm

^{3}H-hypo added: 4/18/97 2:30 pm

Samples processed: 4/19/97 3:00 pm

initial parasitemia: 16%, diluted as follows: 3 mL IBC + 24 mL assay medium + 2.4 mL NRBC

Conclusions

Compound	Stock. conc.	Assay conc.	Percentage of Control
AJ32190	**24.2 mM**	**0.41 mM**	71
AJ32190	242 mM	4.1 mM	7
AJ02030	2 mM	34 μM	8
AJ02030	466 mM	7.9 mM	7
BG01377	5 μM	85 mM	99
BG 01377	50 μM	0.85 μM	106
BL29446	23.1 mM	0.39 mM	96
BJ52052	5 μM	85 nM	10
BH32727	2 μM	34 nM	103

Remarks

Delineation of the IC50 for the compounds showing significant inhibition (AJ32190, AJ02030, and BJ52052) is in progress.

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